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Differential Effects of Estradiol, Estradiol Benzoate and Pregneninolone on *Platypoecilus maculatus.*

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(Plates I-V; Text-figures 1-5).

Androgenic effects of estrogens have thus far been described as affecting only secondary sex organs in mammals (Allen, Hisaw and Gardner, 1939; and Witschi, 1939). In the present work, data are presented to show gonodal stimulation and androgenic effects of an estrogen in the platyfish.

The platyfish, *Platypoecilus maculatus* Günther, is a viviparous cyprinodont belonging to the family Poeciliidae. It has been used frequently for genetic studies (Bellamy, 1928 and 1933; Fraser and Gordon, 1929; Gordon, 1927, 1931, 1937a, 1947a and b), for studies of melanoma (Gordon, 1937b, 1948a; Gordon and Flathman, 1943; Levine and Gordon, 1946), and for embryological studies (Tavolga and Rugh, 1947), but until 1940 it was not used as an experimental animal for endocrinological work.

In 1941, Cohen, Gordon and Nigrelli reported on the spontaneous development of gonopodia in females of *Platypoecilus*, while in 1940 and 1942 Grobstein worked out the development of, and endocrine effects on, gonopodium differentiation. Cohen in 1942 and 1946 did the first work on the effects of sex hormones on the platyfish. At that time he found that pregneninolone has an androgenic effect upon the gonads and anal fins of the fish and that alpha estradiol benzoate has feminizing effects on the male. Pregneninolone, in mammals, has been known as a progestogen, although its effects have been discredited in recent years as ineffective and partially androgenic (Corner, 1942; Freed, 1942 and 1943). As above indicated, it has been found since that time that on lower vertebrates, such as fishes, the hormone has an entirely androgenic effect. In experiments preliminary to the present work, it was found that the effects of free estradiol did not coincide with those produced by the benzoate ester, and it was decided to continue and expand this aspect of the work as well as to determine the effects of androgens on the male and estrogens on the female. Pregneninolone was used as an androgen with which to compare the effects of alpha estradiol when it was found that this substance did not exhibit strictly estrogenic activity.

MacBryde *et al.* (1942) found differential effects of estrogenic substances on the mammalian liver. It was decided, therefore, to investigate the effects of the treatment upon the liver of the experimental animals as a possible source of information as to the reason for the differential effects of the two estrogens. With these purposes in mind the present experiments were undertaken.

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MATERIALS AND METHODS.

The fishes used for these experiments were platyfish, *Platypoecilus maculatus*, of the New York Zoological Society's Genetics Laboratory Culture 180 (Gordon, 1948b), their immediate offspring and those of several subsequent inbred generations. In this strain the males are the heterogametic sex (XY). The Y chromosome carries the "spotted" gene (*Sp*), regularly transmitted from father to son when a spotted male $(X)+/(Y)_{Sp}$ is mated to the recessive female $(X)+/(X)+$. The effect of the *Sp* gene is such that groups of macromelanophores are distributed over the major part of the body. The pigment cells begin to show immediately after the birth of the animals, affording a convenient method of identifying males and females within a day or two after birth. The females carry the recessive gene for macromelanophores (+ or *sp*) and appear as gray. In other strains of the platyfish which do not possess this or a similar feature, sex identification is delayed until such time as the fish mature, when the anal fin of the male is transformed into an "intromittent" organ, the gonopodium. Experiments carried out on such fish necessitate knowledge of the ratio of males to females usually derived in a brood from these fish and the use of statistical methods in determining the deviation from such a ratio when the work involves the use of hormones which produce effects on primary and secondary sex organs. This type of analysis is unnecessary with the strain used.

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In seven generations only one crossover occurred, and this was in the control group. An animal in that group appeared to be a normal female 21mm. long, but on sectioning she was found to possess normal testes for a fish of that age (66 days). Fraser and Gordon (1929) indicated that crossing over of the sex chromosomes in the platyfish is likely to occur at the rate of 1%.

The experimental individuals were kept in two-gallon tanks, approximately eight immature specimens of both sexes to a tank. Plants and gravel were removed at the beginning of each experiment in order to secure more uniform conditions. The room in which the tanks were kept was maintained constantly at a temperature of 80° F., plus or minus 3°.

The fish were fed daily on a fresh liver-Pablum mixture (Gordon, 1943) in amounts such that each tank received approximately 1.5 cc. per week. The method of introduction of hormones received a considerable amount of attention. The literature contains accounts of hormone administration both by dropping hormones into the water and by injection (Berkowitz, 1937; Eversole, 1941; Grobstein, 1942a). It was felt that the animals used in these experiments were too small to receive injections, since at the beginning of treatment they were only two to ten days old and averaged only 8 mm. in length. Therefore, dropping powdered hormone into the tanks was attempted.

It was soon noted, however, that the animals learned early to distinguish between food and hormone. At first they ingested it freely, but after a day or two they were observed to ignore it completely and the powder settled to the bottom of the tank, where it remained until removed. Thus the greater part of the hormone was not utilized. The possibility that it may have dissolved and been absorbed was not overlooked, and experiments designed to check this possibility will be discussed later. However, since the solubility of the hormones is known to be relatively small, another method of more direct administration was attempted, and was found to give good results.

The powdered hormone was introduced into the semisolid liver-Pablum paste and thoroughly mixed. The food was given in small lumps each day and the fish were observed to pick at and ingest it freely each time it was given for the duration of the experiment. They consumed the amount given in about twenty minutes and close observation showed that they did not reject any part of the food, nor could any be seen at the bottom of the tank when later observed. Because of this it was assumed that the entire amount was ingested, and with it all the hormone which did not dissolve in the twenty-minute period which was needed for ingestion. Although no accurate measurement could be made of the amount taken in by any individual fish, it was seen that all the fish had access to the food and that there was little fighting among them for it. Each animal, therefore, received approximately the same amount of food and of the hormone. The hormone in oil preparation was mixed into the liver paste in the same manner, and though it changed the consistency of the paste to a slight degree, the preparation which was chosen contained a high concentration of hormone per cubic centimeter and little of the solution was needed to secure the necessary concentration in the food.

The hormones used were pregneninolone (Pranone, Schering), alpha estradiol (Progynon DH, Schering), and alpha estradiol benzoate (Progynon B, Schering). The first two were prepared in tablet form. The initial supply of estradiol benzoate was in powder form and the remainder in solution in sesame oil. The two forms of estradiol benzoate produced indistinguishable results and so are considered together.

The amounts of hormone used were selected in an attempt to secure doses which would be adequate to gain results and at the same time avoid much of the toxicity which was found to occur, especially from pregneninolone (see Text-fig. 1). Pregneninolone was used in doses of 5, 2.5, 1.25 and 0.625 mg. per 3 cc. of food. Estradiol and the crystalline estradiol benzoate were used in doses of 0.5, 0.25, 0.13 and 0.06 mg. per 3 cc. of food. The estradiol benzoate in oil was obtained in ampules containing 1000 rat units per cubic centimeter of solution in sesame oil, corresponding to 0.166 mg., and the doses given per 3 cc. of food measured 0.166, 0.083, 0.0415 and 0.0275 mg. These dosages were obtained by mixing 1, ½, ¼, etc. cc. of the solution or the same fraction of tablets with 3 cc. of food.

Control tanks containing littermates of the experimental animals were run simultaneously with each experiment. In experiments where oil solution of estradiol benzoate was used, tanks of littermates which were fed with a mixture of food and sesame oil alone were also used.

The doses were given for various intervals, varying from ten days to seven weeks, and at the end of each interval at least one male and one female were removed from each experimental tank and from the control tank. They were immediately fixed in Bouin's picro-formol and preserved after fixation in 70% alcohol. Each fish was then prepared for further study as follows: the anal fin or gonopodium was removed, cleared and mounted entire, and the belly of each animal was slit to facilitate infiltration of solutions. The animal was decalcified in a nitric acid-phloroglucinol mixture for a period of 24 to 48 hours, depending on the size of the fish, placed in several changes of 70% alcohol to remove the acid, and dehydrated by means of Dioxan. After infiltration in 60-62° paraffin, the anmals were cut at 10 μ and the sections stained with Harris' haematoxylin and eosin, or with acid fuchsin and aniline blue (modification of Masson trichrome stain).

The sections thus obtained were examined to ascertain the condition of the ovary or testis, and the general condition of the other organs, and the anal fins were examined for signs of structural changes which might indicate a progressive shift toward maleness or femaleness. The sizes of the gonads were determined by measuring the widths of these glands. It was found that while the lengths of the gonads were roughly correlated with the standard length (length from the tip of the snout to the base of the caudal fin) of the animals, the width of the gonad was also correlated with the development and apparent activity of the gland as induced by hormone treatment.

In order to determine size differences between the eggs of the treated animals and those of the controls, one or more widely separated sections of each treated and control animal was subjected to analysis. The sections selected were as widely separated as possible in order to insure that no duplicate measurements could be made on any egg. Except for this consideration, the sections were chosen at random. In each case, 100 eggs were measured in order to obtain a good distribution and a sufficient and representative sample. The means and standard errors of the means were obtained and according to the formulas given by Simpson and Roe (1939) the significance values were calculated. These results are given in Text-fig. 4 and Table IV. In the case of the testes, the same method of analysis was applied to primary spermatocytes , secondary spermatocytes and spermatophores. In addition, an adult control male was sectioned in order to afford a comparison between the testes of the treated young animals and a normal adult testis. The testis of this animal was analyzed in the same manner.

RESULTS.

Two hundred forty-six animals were used experimentally. Each experimental group contained 26 to 33 animals when finally prepared and sectioned. Since with the dosages used there was no statistical difference between those treated for different periods and with different dosages, the descriptions given will cover all periods of treatment and all dosages, except where otherwise indicated.

General Considerations.

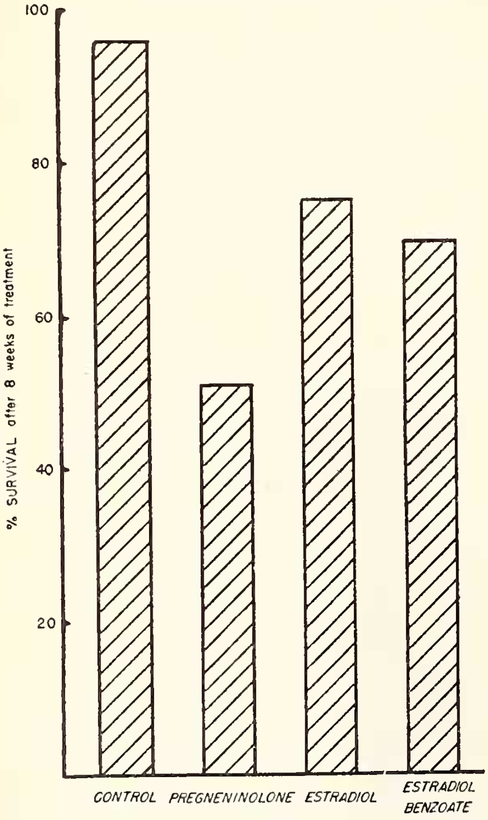
As was stated above, the animals ate the hormone-impregnated food freely and there was observed to be no fighting among the members of any tank for greater amounts of food. Each pellet of food was eaten within about twenty minutes of its introduction into the tank, assuring almost complete introduction of the hormone into the animals. It was possible, however, that the hormone might be entering the animals by way of absorption taking place from the amount of hormone which dissolved into the water during the time when the food was present in the tank. In order to establish whether such dissolution took place, and whether, if it did, the dissolved hormone remained in an active state, two experiments were set up.

In the first of these, the water between two tanks was circulated through glass wool by a conventional air-lift filter in such a way that the water passed from one tank to the other without any undissolved particles passing in either direction. To the immature fishes of the same strain which were placed in the first tank was fed the same liver-Pablum paste as was used for the main experiments. The fishes in the second tank received the same food without the hormone. The water was transferred from one tank to the other at the rate of about 240 cc. per hour, with a complete turnover at the rate of once in every 30 hours. At the end of a week, the fish in the second tank, which received no hormone in their food, had developed the same effects as those in the first tank which were fed directly, showing, first, that dissolved hormones or their metabolic products affected the animals; second, that dissolution had taken place in an amount great enough to produce an effect on the animals; and third, that the hormone was stable during the time taken for the change of water from one tank to the other.

Three weeks to a month after the termination of the main experiments, immature fish were placed in the tanks used for these experiments without changing the water or washing the tanks. The animals showed no effects of any sort and matured into normal adults, showing that after this period of time the hormone was no longer active.

During the main body of the experiments, the effect of the hormones on the experimental animals was first noticed on the males which were treated with pregneninolone. Within four to five days after treatment was begun, when the animals were six to fifteen days old, and measured 8 to 9 mm. in length, each animal's anal fin was modified into the general form of a gonopodium. A few days later, the anal fins of the females in the same tanks had also acquired this characteristic. At about the same time (ten to twelve days), the males in the estradiol tanks developed the same type of modification, also followed in a few days by the females. Note that the estradiol, while it had superficially the same effect, was delayed in its action in comparison to the pregneninolone. Those fish in the tanks treated with estradiol benzoate, both males and females, for the duration of experimentation never developed any structures even superficially resembling a gonopodium. That the gonopodia developed by the androgen-treated animals were not typical nor perfect gonopodia with the characteristic hooks, spines, serrae, etc., of the platyfish gonopodium will be discussed later. It should be said here, however, that they were modified in the male direction sufficiently to be considered greatly affected by the hormone treatment, and that the fins of the pregneninolone- and estradiol-treated animals reacted in the same general manner.

Pregneninolone in the amounts given had a serious effect on the viability of the treated animals (see Text-fig. 1). Forty-eight percent. (32) of these animals died before the termination of the experiment and therefore were not considered in the results given. It is believed, however, that this death rate must be a significant consideration in the general effect of the hormone on the metabolism of the animals, and should therefore be included in the general results. The effect of estradiol was similar, but again quantitatively less, as only 24% (17) of the animals treated in this manner succumbed. This number was significant as compared to the death rate of the control, where there was only 4% mortality, but not as compared to the estradiol benzoate-treated group, since 30% (20) of the estradiol benzoate-treated animals also died.



Text-fig. 1. Percentage survival of control and treated animals over a period of eight weeks.

The locomotor activity of the treated animals was not impaired in any way. Sexual activity, normally absent at this stage of development, appeared precociously, and the tiny animals with miniature gonopodia were seen vigorously following the females, in a manner suggestive of precopulatory behavior of adults, as early as one week after the beginning of treatment, when they were but two weeks of age. This type of activity continued until the end of the experimental period. The females of the group, although they exhibited the male type of behavior, showed it to a lesser degree. Although they tended to follow each other, they did so less often and less vigorously. Females were not seen following males. In the estradiol benzoate tanks there was no evidence of male behavior during the entire course of the experiment on the part of either males or females.

Effect on Gonads.

Control Males.

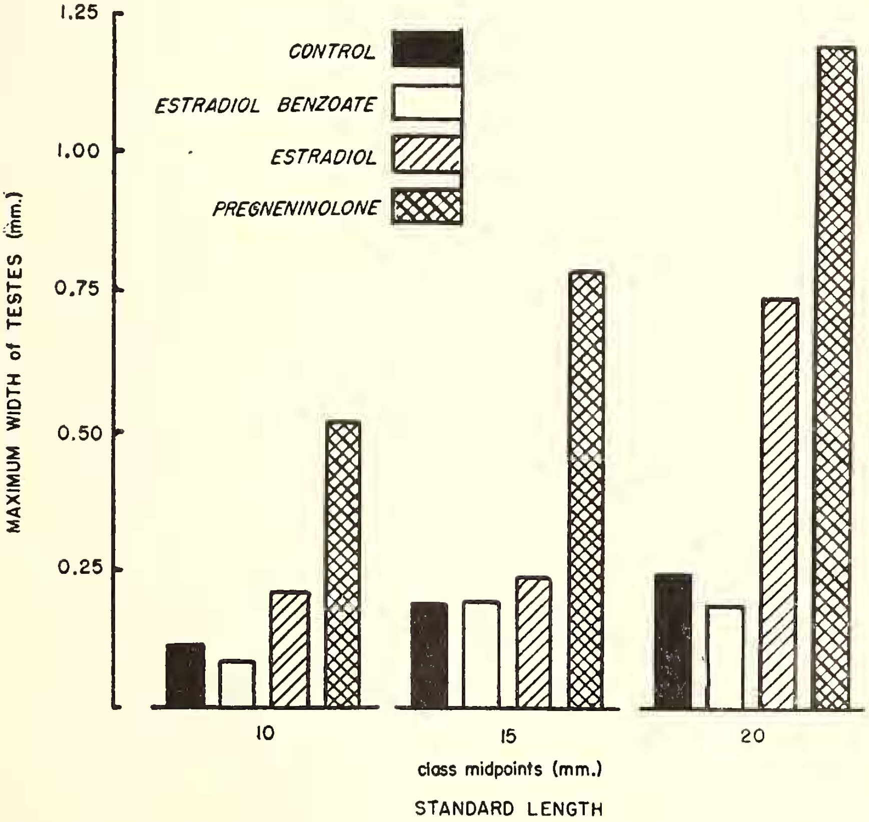
All animals in the experiments were young healthy specimens, ranging in size at the end of the experimental period from 8 mm. for those treated for one week to 22 mm. for those treated for seven weeks. None of the animals at the end of the experiments were old enough to be normally sexually mature, and they would not normally have become so for about two months, as *Platypoecilus maculatus* matures at the age of about four to six months under the laboratory conditions maintained here. At the ages of one to seven weeks, therefore, the testes were small compact masses, fused but showing their primary bilobed nature, their anterior ends appearing between two coils of the intestine at approximately the same cross sectional level as the caudal tip of the liver. They were attached to the dorsal peritoneum by a short mesorchium and in a few cases were seen dorsal to the intestines. See Pl. I, Fig. 1. They ranged in width from 0.08 mm. for the smallest animals (7.0 mm.) to 0.35 mm. for the larger ones (19.0-21.0 mm.). The younger gonads could be seen to contain numerous groups of cells (cysts) which could be only poorly differentiated from the main mass of tissue, except under the higher powers of magnification at which they could be seen to be spermatogonia. Between these cysts existed numerous connective tissue cells and fibers, making up the stroma of the gland. The sperm duct in these smaller animals was poorly differentiated. In the larger specimens, none of which was more than eight weeks old, the cysts were slightly better differentiated. They could be seen to be separated from their surrounding stroma, which was less distinct, and the smaller cysts had now taken up a position relatively peripheral. At this stage these cysts measured 20 to 33 μ in width. A few larger cysts, 36 to 46 μ in width, could be seen toward the center of the gland. These, under higher magnification, could be seen to be primary spermatocytes. These cysts, when present, surrounded the now well formed duct. These descriptions confirm those of Wolf (1931) on the development of the testis in *Platypoecilus maculatus* at this stage. In no case did the gonads contain any cellular formations acceptable as secondary spermatocytes, spermatids, mature sperm or spermatophores.

Males Treated with Alpha Estradiol Benzoate.

The testes of these animals showed a slightly retarded development as compared with the controls (see Pl. I, Fig. 2). Their size range was equal to that of the normals (0.10-0.34 mm.). The mean was 0.19 mm. The two testes were slightly separated, indicating an inhibition of their development toward a fused gland. The gland in general consisted of a number of peripheral cysts, surrounding stroma cells which were abundant and ducts which were slightly less well formed than the normal. The cysts measured 11 to 21 μ in width, but since the testes were so small there were not enough of them to justify a statistical analysis. A few larger cysts were present, but they were less distinctly demarcated than those of the control. There were no statistically significant differences between the widths of the testes of this group and those of the controls (P = .05—see Table II).

Males Treated with Pregneninolone.

Pregneninolone has been shown to have androgenic activity in the guppy by Eversole (1941) and in the platyfish by Cohen (1946). It was expected, therefore, that it would have a similar effect here. That expectation was justified. The testes of even the smallest of the males thus treated showed definite stimulation effects. These testes were significantly larger than those of the control (P = .001), measuring from 0.36 to 1.50 mm., with a mean of 0.81 mm. (see Text-fig. 2 and Table II). In each case, whether the animal was treated for a short period or a long one, the results were the same except for the general size of the gland, which had sufficient time to grow larger in the animals which were treated for a longer period. The cellular effects, in



TEXT-FIG. 2. Growth of control and hormone-treated male gonads as determined by measurements of testis widths.

TABLE I.

Comparative Sizes of Gonads in Treated and Control Females.

Treatment	Number of animals	Mean length	Range	Mean gonad width	Extremes
Control	13	13 mm.	8-19	0.42 mm.	0.11-1.40
Estradiol benzoate	13	17 mm.	8-24	0.31 mm.	0.11-0.61
Pregneninolone	12	16 mm.	13-23	0.45 mm.	0.30-0.85
Estradiol	16	16 mm.	13-23	0.45 mm.	0.20-0.88

every case, were the same. The effect was to stimulate the testes to maturity far ahead of the time at which it would ordinarily be functional. Cysts of spermatogonia were present, measuring 30 to 51 μ in width, but in all the animals of the group the spermatogenic process had gone far beyond the stage of spermatogonia and primary spermatocytes into secondary spermatocytes, spermatids and spermatophores, the presence of which is the usual sign of a functional gland (see Pl. I, Fig. 3). The groups of cells had become differentiated into cysts of maturing primary spermatocytes measuring 93 μ plus or minus 2.1 (see Table III), secondary spermatocytes measuring 97 μ plus or minus 1.5, or later stages, each cyst containing only one stage of spermatogenesis, as is found in mature fish. Many of the cysts contained spermatids in the process of growing tails. Others contained nearly mature spermatozoa with heads embedded in Sertoli cells, beginning the formation of the typical ring of the spermatophore. Still others were found (69 μ plus or minus 1.5 μ) which possessed the completed ring form of the spermatophore, containing mature spermatozoa, tails inward, dark heads forming a ring and massed together. In many cases the released spermatophores were found in the ducts, which is typical of the testis of the mature fish. In all the cases spermatogenesis was active in all its stages; there was an abundance of every stage from the earliest spermatogonia to spermatophores. In a general way the progress of spermatogenesis was from the outer portion of the gland inward toward the duct, and spermatogonial cysts were found mainly at the periphery progressing through primary and secondary spermatocytes to spermatids and spermatophores which were located near the center of the gland and adjacent to the ducts. Since the animals were not treated for more than seven weeks, it is possible that the maximum effects were not obtained. Exhaustion effects in *Lebistes*, in which all spermatogenesis is in very late stages and no spermatogonia are present (Eversole, 1941), were not found. It is possible, therefore, that a longer treatment would have produced glands showing lack of germinal elements such as those described by him.

The position of the glands was also different in the treated fish. In the controls at this age they tended to be placed, as stated before, between the coils of the intestine, and only rarely were situated dorsal to this general position. In the pregneninolone-treated animals, however, the testes had grown so large that they pushed dorsally. They often occupied a position completely dorsal to that of the normal gonad, while in all other cases at least part of the gland projected above the coils of the intestine in cross section. Often they occupied the major part of the body cavity.

The interstitial tissue had also changed. In comparison to the size of the treated gland it was very sparse, being seen as mere threads between the cysts of maturing germ cells. However, toward the center of the gland, where it filled in spaces between the cysts and the sperm duct, slightly more abundant interstitial tissue was often seen. In the main it appeared like connective tissue, often with large oval cells. A few collecting tubules lined with cuboidal epithelium could also be seen.

TABLE II.

Comparative Sizes of Gonads of Treated and Control Males.

Treatment	Number of animals	Mean length	Range	Mean gonad width	Mean W/SL*	t	P
Control	13	15.1 mm.	7.0-21.0	0.20	0.0122		
Estradiol benzoate	12	16.0 mm.	8.5-21.5	0.19	0.0125	.17	.05
Pregneninolone	10	16.2 mm.	8.5-21.5	0.81	0.0518	8.9	.001
Estradiol	17	15.2 mm.	8.0-22.0	0.35	0.0209	2.4	.02

* W/SL equals the ratio of the gonad width divided by the standard length.

TABLE III.

Sizes of Spermatogenetic Cysts of Treated and Control Males.

Treatment	Structure of testis	Number of animals	Sample	Mean diam. (micra)	± σ_M*	General effect
Control at experimental stage	Primary sp. cytes	14				Few—insufficient for significant count
	secondary sp. cytes	14				None present
	sp. phores	14				None present
Control mature male	primary sp. cytes	1	100	60	1.4	
	secondary sp. cytes	1	100	73	1.3	
	sp. phores	1	100	49	.9	
Estradiol benzoate	primary sp. cytes	12				Very few
	secondary sp. cytes	12				None present
	sp. phores	12				None present
Pregneninolone	primary sp. cytes	10	100	93	2.1	Enlarged over mature control
	secondary sp. cytes	10	100	97	1.5	Enlarged over mature control
	sp. phores	10	100	69	1.5	Enlarged over mature control
Estradiol	primary sp. cytes	2	100	56	1.4	Not significantly smaller than control
	secondary sp. cytes	2	100	56	1.1	Significantly smaller than control
	sp. phores	2	100	42	1.1	Significantly smaller than control

Significance Values Calculated from Means in Table III.

Significance values are calculated as the difference between two means divided by the standard error of the difference.

Primary spermatocytes

	Estradiol	Pregneninolone
Control mature male	2.0†	13.0
Pregneninolone	14.8	

Secondary spermatocytes

	Estradiol	Pregneninolone
Control mature male	9.9	12.1
Pregneninolone	22.2	

Spermatophores

	Estradiol	Pregneninolone
Control mature male	5.0	11.2
Pregneninolone	14.6	

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* σ_M equals standard error of the mean.

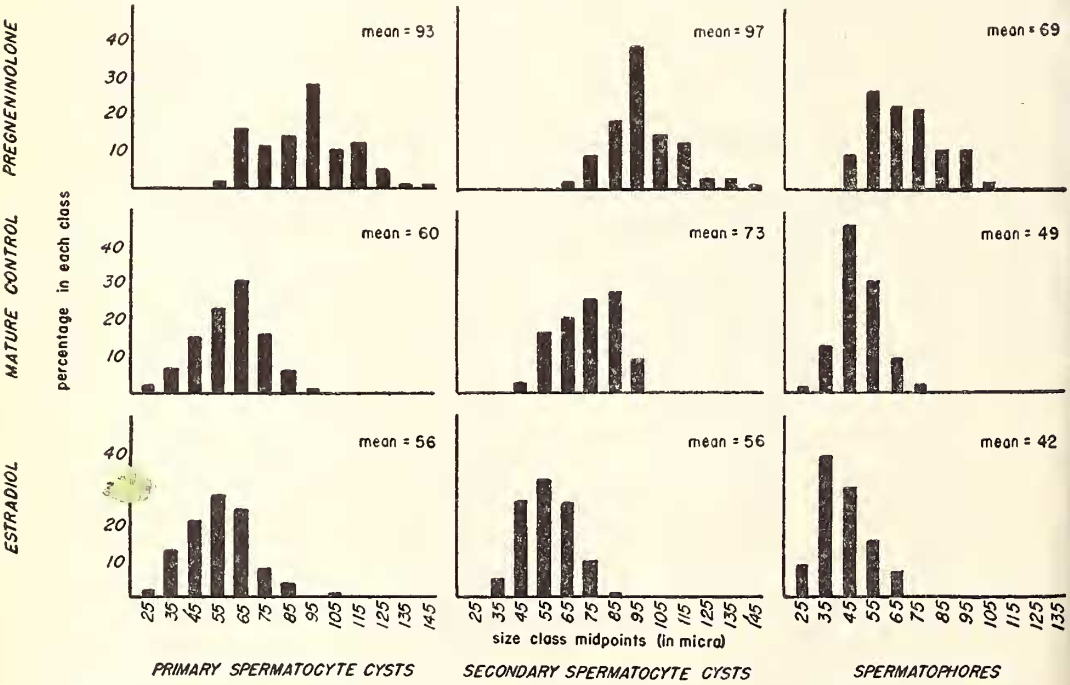
† These values are not to be considered significant.

These observations corresponded very well to Wolf's (1931) description of the interstitium of a young mature male.

Males Treated with Alpha Estradiol.

In mammals it has been found that alpha estradiol is usually an estrogenic hormone (Willier, 1939; Witschi, 1939), although in some cases paradoxical effects have occurred which have affected the secondary sex organs. However, the gonads are not ordinarily changed in these cases. When the present experiments were in their earliest stages, it was found that apparently this was not true in the platyfish. Therefore further experiments were carried out in order to determine the effects of this substance. The animals in this group fell into two sets, the difference being one of size and depending not at all on dosage or length of treatment. In all animals under 18 mm. in length, the testes presented a normal control picture. The testes were small, compact, showed spermatogonia and early spermatocyte stages, compared well in size with those of the controls, except for a very small increase, and generally showed no significant effects. In all animals, however, over 19 mm. in length, the developmental picture was entirely changed. The gonads in these cases were intensely stimulated organs, showing all stages of spermatogenesis. Discrete cysts of primary and secondary spermatocytes, 56 plus or minus 1.4 μ, and 56 plus or minus 1.1 μ, respectively, spermatogonia 25 to 30 μ at the periphery, and normal spermatophores of 42 plus or minus 1.1 μ at the center were present. Note that the sizes of these cysts were significantly smaller than those of the pregneninolone animals (see Text-fig. 3 and Table III). The ducts also were large and well formed, typical of the ducts of a mature male, and they were frequently filled with spermatophores. These spermatophores, like those of the pregneninolone-treated animals, were well formed and showed no sign of precocious extrusion from the cysts or of abnormal cells despite their comparatively small size (see Pl. I, Figs. 4 and 5). The picture approximated that of animals treated with the known androgen pregneninolone, in every detail except size. It seems, then, that estradiol, far from being an estrogen, acts much like an androgen in the stimulation of the testes in these fish. The position of the testes in the abdominal cavity of the animal and the appearance presented by the interstitial tissue corresponded in every way to the pregneninolone-treated animals.

In order to determine the size relationship of these stimulated spermatogenic cysts to those of the normal testis, an adult untreated male was sectioned and prepared in the same manner as the experimental animals. As with the experimentals, 100 cysts of each type, primary and secondary spermatocytes and spermatophores, were measured by means of the ocular micrometer and statistical analyses were made (see Table III and Text-fig. 3). In each case the cysts of the pregneninolone-treated animals were significantly larger than those of the adult control and



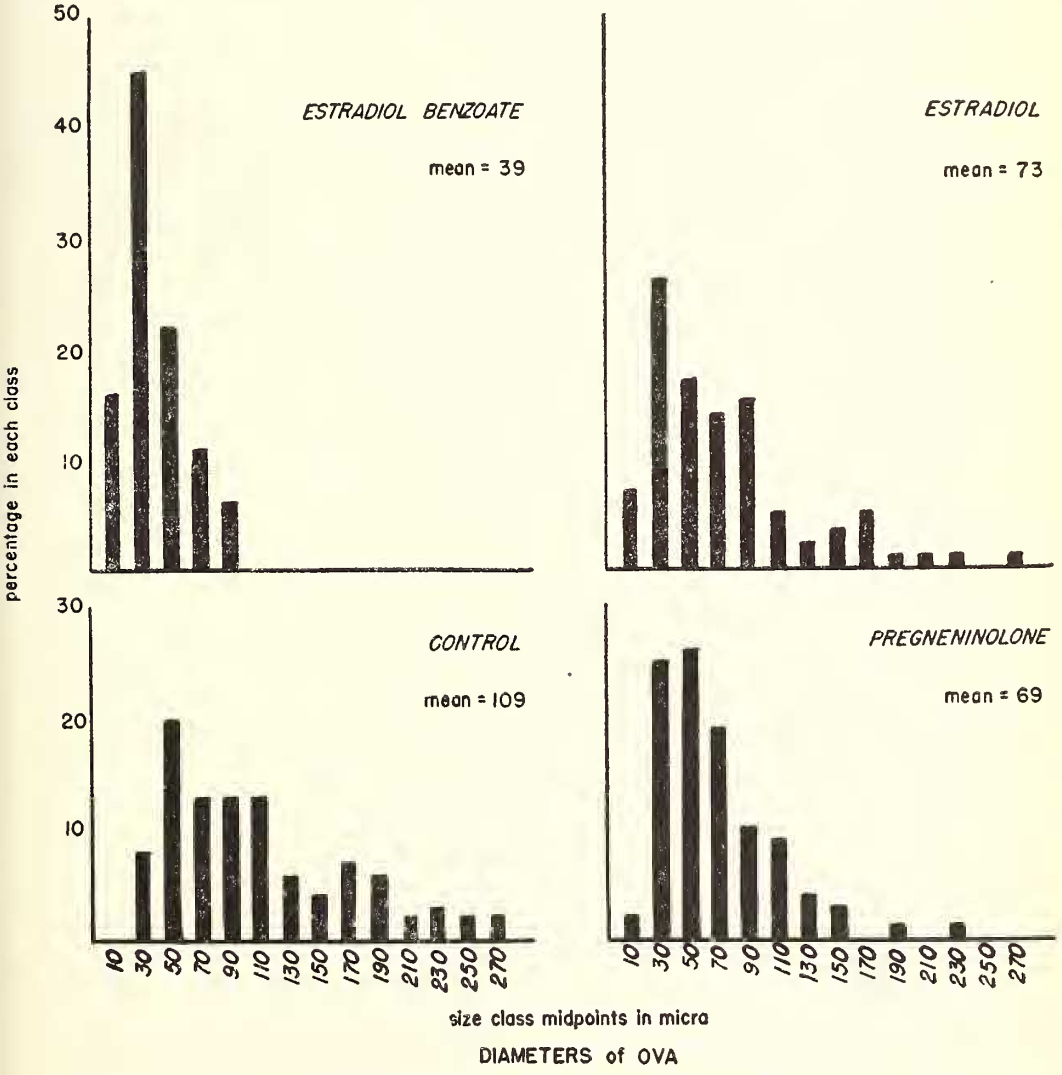
TEXT-FIG. 3. Comparative size ranges of primary and secondary spermatocyte cysts and spermatophore cysts of pregneninolone- and estradiol-treated animals as compared with those of a normal adult male.

with one exception the cysts of the estradiol-treated animals were smaller. In the case of the primary spermatocytes the cysts of the estradiol-treated animals were not significantly smaller than those of the control adult.

Control Females.

The typical female ovary of a platyfish of two to eight weeks of age was located in serial cross sections between two coils of the intestine, suspended from and approximating the dorsal peritoneum. The ovary ranged in size from 0.11 to 1.40 mm. and the mean value was 0.42 mm. Posteriorly it lay free of contact with surrounding organs, and far posteriorly, just anterior to its posterior margin, it lay almost if not completely free in the abdominal cavity. At the young stages studied here, it consisted of a single mass composed mainly of young circular or ovoid ovocytes, before, or in the larger animals during, the process of yolk deposition. The mean size of these ovocytes was 109 μ plus or minus 6.5 μ (see Text-fig. 4 and Table IV). The ovary as a whole was compact, little if any space being present between adjacent eggs. Any space which was present was almost filled with small amounts of stroma. Stroma also filled spaces between the outermost eggs and the peripheral flattened epithelium (see Pl. II, Fig. 6).

In the younger stages the ovocytes were yolk-free, with slightly reticular cytoplasm and a lighter-staining nucleus. Each nucleus contained one or two deeply-staining, prominent nucleoli. The nucleoplasm itself was reticular in appearance, studded with chromatin granules. These larger eggs were located mainly at the periphery of the organ and each was bounded by epithelial cells constituting the follicle. The younger cells, oogonia, 21 to 45 μ in diameter, were situated



Text-fig. 4. Comparative size ranges and distributions of eggs of control, estradiol benzoate-, pregneninolone- and estradiol-treated females.

TABLE IV.

Sizes of Eggs of Treated and Control Females.

Treatment	Number of animals	Sample	Mean diam. (micra)	$\pm \sigma_M$	General effect
Control	12	100	109	6.5	
Estradiol benzoate	13	100	39	2.1	Great inhibition
Pregneninolone	12	100	69	3.9	Partial inhibition
Estradiol	16	100	73	4.8	Partial inhibition

Significance Values Calculated from Means in Table IV.

	Control	Estradiol benzoate	Estradiol
Pregneninolone	5.2	6.7	0.6*
Estradiol	4.3	6.1	
Estradiol benzoate	10.2		

* These values are not to be considered significant.

nearer the ovarian cavity. They were sometimes imbedded in the stroma immediately surrounding it, but usually maintained contact with the epithelium of the cavity. Their cytoplasm was more deeply stained and presented a more homogeneous appearance.

In the later stage the situation was much the same except for the appearance of the larger eggs. These had now grown considerably in size, measuring 100 to 280 μ, and their cytoplasm presented a far more reticular appearance than was true of the younger eggs. Near the periphery of some of them yolk granules were discerned, but this was true only in the largest of them. In all cases intermediate stages were present between the largest and smallest eggs.

Females Treated with Alpha Estradiol Benzoate.

The ovaries of the animals treated with alpha estradiol benzoate were more compact and smaller than those of the controls, measuring 0.11 to 0.61 mm. and averaging 0.30 mm. in width. The eggs appeared tightly pressed together and were deformed by this pressure in some cases. The ovaries were more closely pressed between the coils of the intestine. Although the arrangement of the eggs appeared normal and a fairly well formed duct was present, the eggs themselves showed an inhibition of development (see Pl. II, Fig. 7). The larger eggs were peripheral, gradating to smaller ones in the center of the organ. In size they ranged from 11 to 96 μ (see Text-fig. 4 and Table IV), and none of them approached the size of eggs found in the control fishes of the same size. No evidence of yolk deposition was present in any of the eggs in this group. The cytoplasm of these eggs was more homogeneous than was true of the ova of the controls, and they compared in size and structure to a much younger stage than that which would be expected from the age and size of the fish. It is evident, then, that though few acute abnormalities were present in the structure of the individual eggs, their size and appearance indicated that they were greatly inhibited by the treatment.

Females Treated with Pregneninolone.

The ovaries of these animals were again slightly smaller than those of the controls, measuring from 0.30 to 0.85 mm. and averaging 0.45 mm. in width. The sizes of the groups overlap a great deal (see Table I), but the largest ovaries of the pregneninolone-treated animals did not reach the size of the largest ovary of the control group. The main effects, however, were those appearing in the size and structure of the eggs. The ovaries of these animals presented an extremely abnormal appearance. The greater number of them were shrunken and small, appearing completely pressed out of shape by the surrounding coils of the intestine, as in the estradiol benzoate-treated animals (see Pl. II, Fig. 8). It is doubtful, however, whether this shrunken appearance was due to the pressure caused by the intestine, since the control ovaries were subjected to the same pressures and did not show the deformities. Also the pregneninolone ovary, like the controls, lay, at its posterior end, free in the abdominal cavity, and the deformities were equally great there. Therefore some other cause must be assigned to this phenomenon, presumably one due to the hormones involved. The ovaries contained in most cases little interstitial tissue, and while this was more deeply stained than the normal, it did not appear especially abnormal. The eggs themselves, however, showed definite effects. They were seldom as large as those of the control, having a mean size of 69 μ plus or minus

3.9 μ (see Text-fig. 4 and Table IV). In a few cases large eggs could be found and these were the most nearly normal-looking ones. Even they appeared degenerate, however, showing deeply-staining cytoplasm, slightly irregular nuclei and a partially deformed appearance. The remaining eggs were uniformly deformed in shape, the main body of them having irregular depressions in their sides and usually one concave side, so that the individual eggs took on the appearance of pushed-in balls. The nuclei were also misshapen, showing elongations and irregularities, each one staining deeply. The cytoplasm often had a mottled appearance, in contrast with the even staining of the control. This resulted, presumably, from some effect on the cytoplasm, which caused parts of it to stain deeply and others lightly, without any regularity. Another significant point was in regard to the size of the eggs. All those which were not included in the groups of larger eggs mentioned first were extremely small as compared to eggs in the same stage of a control, measuring from 11 to 60 μ. No evidence of yolk deposition could be seen in any individual eggs.

In most of these ovaries the duct was poorly formed and the edge of the epithelium was ragged and abnormal, showing cells and bits of tissue sloughing off into the duct.

Two variations of these conditions were found. In two cases the ovary was large but the eggs were scattered and large spaces were present between them (see Pl. II, Fig. 9). While the eggs in these specimens were not usually as deformed as they were in the cases described above, they were deeply stained and appeared to be in a state of degeneration. As above, few eggs could be found which were as large as those of the control of the same age and size, but several appeared which had been approaching this size and condition before treatment with hormones was begun. These eggs showed approximately the same irregularities as the large ones described above. The epithelium surrounding the gland was ragged and shredded in many places and the cells of the duct were ragged.

In two other cases a definite bi-partite ovary was found. In one of these the eggs were fairly large and normal-looking, measuring between 90 and 130 μ (see Pl. II, Fig. 10), and appeared to be comparable to the eggs of the majority of the control ovaries. In the other case, however, the eggs were small and degenerate-looking, measuring about 40 to 60 μ, and were stained deeply, showing deformities. In this ovary there appeared two definite ducts, one for each half of the gland, which showed fairly regular epithelium. The first case contained a duct which was wide and flat horizontally, apparently serving both sides of the gland. It is believed that this remaining evidence of the bi-lobed nature of the embryonic gonad may have been caused by the inhibitory effects of the androgenic hormone applied. All variations of the ovarian conditions were used together in making the statistical analysis of the eggs in this group.

Females Treated with Alpha Estradiol.

In general terms, the ovaries of this group showed the effects expected of an androgen. The results were very similar to those produced by pregneninolone. The majority of the animals possessed ovaries which appeared as shrunken masses, with deformed eggs such as those described for the pregneninolone-treated animals, staining poorly and in a mottled fashion. The size also, of both the ovaries and the eggs, was comparable to the size of those of a pregneninolone-treated female, since measurements of the ovaries ranged from 0.20 to 0.88 mm. with a mean size of 0.45 mm. (see Table I). The eggs had a mean size of 73 plus or minus 4.8 μ, a size which is not significantly different from that of the pregneninolone-treated eggs.

There were several exceptions to this general picture. In two cases the ovary showed the same scattering which appeared in some of the pregneninolone-treated animals (see Pl. II, Fig. 11). The same larger degenerating eggs, and the same type of atretic appearance in the small eggs was present. In one case, there appeared a bi-lobed ovary such as that described above, which possessed one duct to serve both parts of the gland. In this specimen the eggs were small, measuring 40 to 60 μ, and while not as deformed as the typical eggs of this group, some atypical shapes were present and the eggs generally stained more deeply than the controls. In some cases there appeared a variety of degeneration not seen in the pregneninolone-treated group. In these ovaries there were a few eggs which appeared to be almost normal, both in size and general appearance. The remainder of the comparatively large gland was composed of a substance which at first appeared to be adipose material. Upon closer inspection, however, it was concluded that at the places where this material was seen, there had once been large eggs in the process of yolk deposition (see Pl. II, Fig. 12). The eggs had apparently degenerated, since the masses contained no recognizable structures, and left behind them a mass of fatty yolk-filled material. Vacuoles were present, which showed the presence of lipoids. Some yolk granules were to be seen. At certain points about the periphery of these masses there appeared epithelium of a largely degenerate nature which was broken and sloughed in parts. Since there had been several eggs of this nature, it might have been expected that there would be some type of separation between them. For the most part, however, this was not true, and the masses were indistinguishable from one another, showing no evidence of where one egg ended and another began. In some places a portion of the above-mentioned epithelium remained, to give some indication of the limits of the egg, but this was true only in a few cases. The masses of material were of various shapes so that no indication remained of the original shape of the ovum. The remaining eggs were of the small deformed type mentioned above and were pushed to one side of the organ. The ovary, because of this peculiar content, was quite large, although the egg content was extremely small. The appearance of the organ as a whole was a degenerate one. The outline of the organ, even where the eggs were present, was ragged and appeared degenerate, as the epithelium was ragged and uneven in contrast to the smooth epithelium of the control.

Effects on the Anal Fin.

Grobstein in 1940 published a complete description of the developmental stages in the transformation of the platyfish's anal fin into the gonopodium. In 1942, however, he partially changed the terminology used in order to conform to prevailing taxonomic usage. In all the following descriptions, the terminology used in the 1942b paper will be employed.

Control Males.

The anal fin of the control male fish at the ages studied here was one in which no differentiation or growth had taken place in the change from the undifferentiated fin toward the typical gonopodium. The fin looked like the female fin of the same age (see Pl. III, Fig. 13). The fins were well formed, the third ray slightly thicker than the others. The fourth and fifth rays, particularly the fourth, projected slightly beyond the others. No bifurcations were present in the younger fins and the larger ones possessed only primary bifurcations. Secondary and tertiary bifurcations which, according to Grobstein, depend on age, had not yet appeared in any of the fins studied. No growth of the third ray, which indicates the beginning of differentiation into the gonopodium, had begun in any of the fins. The only difference between the male and female control fins at this age was the presence of macromelanophores in the male fin, due to the *Sp* gene. The females did not possess these macromelanophores.

Males Treated with Alpha Estradiol Benzoate.

Fins of the males treated with alpha estradiol benzoate presented the same picture as did the controls. In all the fins, which came from animals not more than eight weeks old, no differentiation of any sort tending in the male direction was seen. The fourth and fifth rays projected slightly beyond the others, but no more so than is normal in the female fin and certainly not enough to give the impression that they are beginning the gonopodial growth phase (see Pl. III, Fig. 14). They appeared as normal fins for this age of fish, but since no animals were carried to maturity it is not known whether the hormone would have been enough to prevent gonopodial differentiation entirely.

Males Treated with Pregneninolone.

The anal fins of all animals in this group were affected by the hormone treatments. In the case of the smallest animals, ten days to two weeks of age and 8 to 9 mm. in length, which had been treated for one week to ten days, the transformation had proceeded only into Phase I, and all these fins possessed third rays which were segmenting and growing, producing an elongation of the cephalad portion of the fin. All those treated for three weeks or longer, however, showed a completely modified picture. In these groups, every fish possessed an almost completely differentiated gonopodium (see Pl. III, Fig. 15). Most of these were almost perfect, although a few existed which were lacking in certain elements present in a normal fin. The usual element missing in such an incomplete fin was the spines, which in most cases, if present, were flattened and smaller than normal. Some fins were seen where no spines at all were present. Since the oldest fish in the group were not more than eight, or at most, nine weeks old, it can be assumed that this precocious development was due to the effects of the hormones administered. That the modifications of the fins correlated well with the growth and differentiation of the gonads is further evidence for this assumption.

Males Treated with Alpha Estradiol.

Anal fins of males treated with alpha estradiol could be placed in two groups. These corresponded directly to the division which occurred in the description given already of the gonads of this group. In those animals which were below the size of 18 mm. at the end of the experiments, the fins, like the gonads, did not show the usual effect of the androgen. Each of the anal fins observed in this group was in Phase I of growth and elongation of the third ray. The fins appeared as modifications of the female condition in which the third, fourth and fifth rays had grown long enough to project beyond the others to about one-third of their length. In the majority of the animals, the third ray was found to be segmented as in Phase I, rather than like the control, in which segmentation was far less definite.

The group comprising those animals which reached a size of 19 mm. or more contained fins which were modified far more toward the typical male condition. They were almost complete but showed more variability than did the pregneninolone-treated males. Elements which were present consistently were the subterminal segments with the terminal hook, the elongation of the fourth ray, with its cephalic ramus curved in a cephalad direction, spines and proximal serrae. Elements which were absent or incomplete in the imperfect fins were distal serrae, the spoon and spoon support, and the blade. These were absent in different combinations. Although the fins of this group were not complete, they showed a definite tendency toward the male form (see Pl. III, Fig. 16). If, as Grobstein postulates, the gonopodium is under the control of the testis, the present evidence supports that view.

Control Females.

The anal fin of the female control animals, like the males at this age, showed no signs of differentiation toward the adult form. The structures possessed by these fins were those typical of the young female (see Pl. IV, Fig. 17). The fins did not differ materially from the adult female type except that, as in the male, only primary bifurcations of the fourth to ninth rays had taken place. The third, fourth and fifth rays projected slightly beyond the others, as is normal. The thickening of the third ray present in the male fin at this age was present to a lesser degree in the female. No macromelanophores were present, since the female does not carry the *Sp* gene. Except for this last distinguishing characteristic, present only in this strain, the fins were structurally similar.

Females Treated with Alpha Estradiol Benzoate.

In animals treated with this estrogen, the condition of the anal fin was indistinguishable from that of the control fin (see Pl. IV, Fig. 18). The third, fourth and fifth rays showed the same slight extension. The third ray was again slightly thicker than the others. Bifurcations of the rays in animals of the same age were identical.

Females Treated with Pregneninolone.

The fins of the animals in this group presented a varied picture. None of them possessed a complete and normal gonopodium, but neither did any possess the typical female anal fin. All animals possessed fins which had progressed far beyond the first phase of gonopodium formation and many had gone into the third phase (see Pl. IV, Fig. 19). All animals had completed the preliminary growth phase, in which the 3, 4, 5 ray complex segmented and pushed out in the cephalo-distal portion of the fin to form a promontory there. Elongation of these rays continued throughout Phase II of gonopodium formation. At the beginning of this growth new segments appeared in the third ray, and at the end of the first phase there were generally nine segments present (Grobstein, 1940). During the second phase, these segments increased in number to twenty-two when the gonopodium had completed its growth. All animals in the group possessed at least ten segments and specimens were found in which the complete number was present. At the end of Phase II differentiation of the various specialized parts of the gonopodium began. The great majority of the animals had arrived at this stage. In many, however, the differentiation was aberrant, showing certain completely differentiated parts, while other parts, which should have differentiated concurrently, were still in an undifferentiated or partially differentiated state. Plate IV, Fig. 19, shows a gonopodium of this group in which the differentiation was almost complete. This fin had progressed as far as the "blade stage" (Grobstein) and shows most of the elements of a complete gonopodium in a more or less normal state. The fin possessed proximal and distal serrae, the blade, the spoon and spoon support, the terminal hook and subterminal segments and other elements. Other gonopodia were found which possessed good segmentation of the third ray and a perfect terminal hook, which should not appear until the time at which the distal serrae differentiate and after the formation of the proximal serrae, but both sets of serrae were missing. Such varied differentiations were common but the general rule in this group was partial or complete differentiation of all parts, many with slight deformations.

Females Treated with Alpha Estradiol.

Alpha estradiol was found to have almost the same effects as pregneninolone, although they were somewhat delayed (see Text-fig. 5). Thus the majority of the fins in this group had begun differentiation but had progressed to a lesser degree than those of the previous group. All the fins had entered the first or preliminary growth phase, since all of them showed at least the segmentation and strengthening of the third ray, and the resulting promontory forming on the cephalodistal border. Most of them had in addition entered the second phase, in which growth had gone on to form a great elongation of the fin. About one-third of them went on into the third phase, in which they showed various stages of differentiation. In this group no complete gonopodia were found, but many fins showed the beginnings of the differentiated elements. There were present terminal hooks together with subterminal segments, proximal serrae, a few sets of distal serrae, the cephalic turning of the fifth ray, and the cephalic ramus of the fourth ray. All these, however, while they appeared together in a few animals, were usually present in less complete combinations. Alpha estradiol, while it is androgenic to a great extent, since it induces the formation of gonopodia, does not produce nearly as complete gonopodial structures as does pregneninolone (see Pl. IV, Fig. 20). The presence of an androgenic effect, however, is easily seen.

Effects on the Liver.

Control Animals.

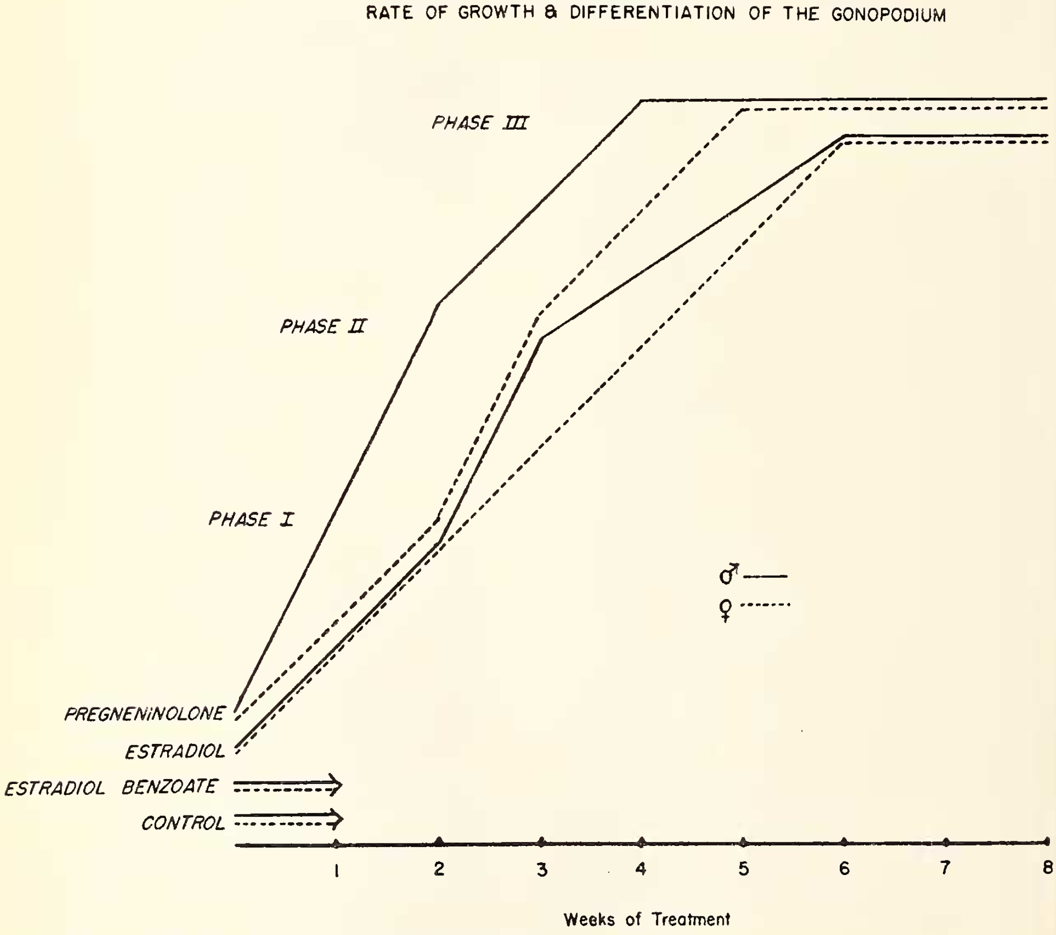
The liver in the platyfish was divided into several large lobes. Lobules were not separated as in the mammal, and the entire gland appeared as a compactly arranged mass of cells. Cords of cells were present, though not as distinctly as in the livers of higher forms. The cells themselves measured about 8 μ in diameter and were weakly eosinophilic. The nuclei averaged about 2 μ in diameter. The stroma was poorly distinguished from the epithelial cells. The mass of homogeneous-appearing cells was profusely supplied with blood vessels of all sizes. The nuclei, from 2 to 3 μ in diameter, were nearly circular and thickly granular. The cells were of three types, non-vacuolated with weakly eosinophilic cytoplasm, partially vacuolated with one or two vacuoles filling about one-third of the cytoplasm, and a third type in which larger vacuoles filled most of the cell. In all the vacuolated cells, the vacuolated ends aggregated together in a direction farthest from the blood vessels, producing pale blotches in the structure of the liver from 10 to 20 μ in diameter and very irregularly placed. The predominant type of cell was the partially vacuolated one. The second cell type in order of predominance was the one which was non-vacuolated and weakly eosinophilic. See Pl. V, Fig. 21.

Animals Treated with
Alpha Estradiol Benzoate.

The liver cells of these animals averaged 8 to 12 μ in diameter. Three types of cells were present, one non-vacuolated and weakly eosinophilic, another containing a vacuole which filled one-third to two-thirds of the cytoplasm, and a third which was highly vacuolated. The predominant type was that in which one-third to two-thirds of the cytoplasm was vacuolated, followed by the non-vacuolated type. The organ as a whole presented a compact appearance much like that of the control. The stroma network resembled that found in the control. No degenerative effects could be noted. See Pl. V, Fig. 22.

Animals Treated with Pregneninolone.

The cells of the livers of these animals averaged from 10 to 15 μ in diameter, being generally much larger than those of the control. The cells were all more or less vacuolated. The type of cell which predominated was one in which the cytoplasm was occupied almost completely by a large vacuole. The vacuoles occupied, on the average, about nine-tenths of the cytoplasm and as a direct result the nucleus and remaining cytoplasm were pushed to one side. The general arrangement of the cells about the capillaries was as in the control and the entire organ presented a highly vacuolated appearance (see Pl. V, Fig. 23). Vascularization appeared increased, with a capillary for every



TEXT-FIG. 5. Graph showing growth of gonopodium of control, estradiol benzoate-, pregneninolone- and estradiol-treated animals.

seven to ten cells. The stroma network appeared more distinctly as a result of the extreme vacuolization of the parenchyma cells. The entire organ showed changes in structure, fatty in nature as demonstrated by the application of Sudan IV in frozen sections. The controls used for these reactions were normal livers of the same age, which showed comparatively few sudanophilic globules. The experimental animals, on the other hand, showed an abundance of these globules in their liver cells.

Animals Treated with Alpha Estradiol.

The liver cells in these animals averaged about the same size as those of the estradiol benzoate-treated animals, but the great majority of them showed vacuoles. Occasional cells showed the extreme vacuolization of the pregneninolone-treated livers, nine-tenths of the cell being occupied by a vacuole. For the most part, however, the cells contained vacuoles which occupied about one-half their volume and there appeared frequently cells with several small vacuoles instead of one large one. In general, the organ showed signs of far greater vacuolization than the control, and, therefore, partial changes similar to those shown by the livers of the pregneninolone-treated animals, but in a stage which was far less advanced. See Pl. V, Fig. 24.

DISCUSSION.

Regnier in 1938 made a comprehensive survey of the history of fishes as experimental animals in sex hormone studies, and in addition described her own experiments on *Xiphophorus hellerii* with anterior pituitary hormones and ovarian and testicular powders and extracts. Essenberg (1926) described sex reversal in *Xiphophorus*. Blacher in 1926 found that testicular hormones are necessary for secondary sex characteristics in *Lebistes*. Castration and implants of gonads in *Xiphophorus* was carried out by van Oordt and van der Maas (1926). Berkowitz, in a series of papers (1937, 1938, 1941a and b), described the effects of estrogens and mammalian gonadotrophins in *Lebistes*, while Eversole (1939 and 1941) worked on the effects of androgens in this animal. In two papers in 1941 (a and b), Turner tested the effects of androgens on *Gambusia*. Scott (1941 and 1944) worked on the effects of steroids on the skeleton of *Lebistes*.

Eversole (1941) mentioned that testes of *Lebistes* treated for 42 days with pregneninolone showed all stages of spermatogenesis, with later stages predominating, that the spermatids and spermatophores produced were abnormal, and that in animals treated for 50 days, later stages in spermatogenesis were present almost exclusively, spermatids and spermatophores were atypical and that the gonad was generally in a degenerate state because of a rapid maturation of the germinal elements which left few spermatogonia. He also stated that the epithelium of the ducts tended to dismember at that time and that the stroma was hypertrophied. These observations were not seen in the present studies. Most of the animals, it is true, were not treated for more than six weeks, but some were treated for seven weeks, and even these did not show the degeneration of the gonad which was described by Eversole. Whether this difference was caused by the difference in experimental animals used, by the different method of administration of the hormone, by differences in dosages or ages, or by different conditions under which the animals were kept, is not clear. However, the gonads of the animals in the present work, while they showed great stimulation, were seen to contain germinal elements and abundant primary and secondary spermatocytes as well as spermatophores. This was true whether the animals were treated for two weeks or for seven. In the case of the animals treated for six to seven weeks, the picture of the gonad was equivalent to a normal mature testis, both in size and in quality of the elements contained. Spermatogonia were present, both primary and secondary spermatocytes were abundant, and the later stages of spermatogenesis were abundant and appeared normal. No signs of degeneration of any sort could be distinguished. As was stated in the results, it is possible that a longer period of treatment might have produced the exhaustion effects spoken of by Eversole.

Winge (1934) has shown that sex reversal may be detected by genetic means in the guppy. These reversals, however, were always from female to male. It is evident, however, that the sex determining mechanism in the guppy is less stable than that of the platyfish, since only two cases of sex reversal in *Platypoecilus* have been mentioned in the literature. Both of these were naturally-occurring phenomena (Breider, 1942; Gordon, 1947a). No sex reversal in the platyfish has ever been reported as having been induced by hormonal or any other means. Because of this relatively unstable sex mechanism in the guppy, it is easier to understand why Berkowitz (1937) was able to secure sex reversal and ovotestes in this form. No such phenomena were found in the present work. Although degeneration of the gonads was common as a result of hormone introduction, no sign of any transformation in the gonads was obtainable, either from male to female, or from female to male.

Berkowitz (1937, 1938, 1941), in work on the guppy, mentioned several hormones and combinations of these which were administered, and combined the results into a general statement. It is possible, therefore, that the divergent results of one or more of the hormones given by him went unnoticed because of this procedure. Although the results appear to be consistent, no mention is made of exactly which results were occasioned by which hormone and slight differences which might have led to a suspicion of the present findings might have been overlooked. A regrettable tendency evident in many papers is to administer "estrogens" without regard to which estrogen is being administered. The present work indicates that such a procedure is not safe.

Essenberg in 1923 stated that the oviduct in *Xiphophorus* was derived from a fusion of the two embryonic components of the ovary in such a way as to leave a space between them which later developed into the oviduct. Wolf (1931), on the other hand, who worked out the embryology of the gonads in the platyfish, stated that the oviduct originated by what may be considered the classical method, the degeneration of the medulla of each embryonic gonad accompanied by the development of the cortex (Willier, 1939). Goodrich *et al.* (1934) found that the oviduct of the guppy originated in the same way. Two such opposing views in two forms which are comparatively closely related seems to be unusual. Evidence for the double origin of the oviduct, in which the duct develops in two parts, one in each embryonic ovary, fusing to form one duct when the ovary itself fuses (Wolf's version), is given by the occurrence of the degenerate ovary under androgen treatment found in this work which possessed two distinct ducts. Such a condition, under the terms of Essenberg's hypothesis, would be unlikely. A further investigation into the origin of the oviduct in *Xiphophorus* would seem to be in order.

Regnier (1938), in her description of the origin of the oviduct in *Xiphophorus,* quoted Essenberg, but since this phase of her work was a review of the literature, no further evidence was to be found there. Regnier mentioned the effects of testis powder as producing bi-lobed and retarded ovaries in *Xiphophorus* when these animals were treated when very young. She also mentioned the comparatively great mortality present when this treatment was given, but said that with the addition of anterior pituitary lobe powder to the water in which treated individuals were placed, the mortality markedly decreased. After injections of testosterone for two months, her animals showed mature sperm in the testes, but no mention is made of presence or absence of spermatophores. Therefore it is not known whether the treated males in that group were fertile. She also discussed sex reversal due to hormones and the prevention of sex reversal by injections of appropriate hormones, but since it is known that the sex determining mechanisms of *Xiphophorus* are somewhat labile (Essenberg, 1926; Witschi, 1939), these results are not inconsistent. Mention was made of certain residual bodies which were derived from the degenerating follicles of the sex reversing ovaries and which traveled to nearby organs where they established themselves. Although evidence of such bodies was sought in the surrounding organs of the fish in the present work, no results were obtained.

Cohen in 1942 and 1946 treated female platyfish with pregneninolone and males with estradiol benzoate. He found at that time that estradiol benzoate had feminizing effects on the male platyfish over a twelve-week period. The other results produced were similar to those found in the present experiments within the time limits used here, except that Cohen showed evidence that mature ova were found in normal control ovaries of fishes only eight weeks old. In the entire group of control females used in the present work, only one such ovary was found. This lack of yolk-filled eggs in the ovaries was not considered unusual, since, although growth rates vary with environment, feeding and other factors, *Platypoecilus*, even under ideal conditions, does not usually mature until the end of the fourth month after birth or later, as will be shown. Under normal conditions, no mature ova would be expected to occur until or just before that time. The effects of pregneninolone which were repeated in the present experiments were in the main more pronounced than those shown in Cohen's work, probably because of the larger amount of hormone actually introduced into the fish as a result of the different method of administration used here. It is believed that this method has been more effective, since the main portion of the hormone was introduced into the fish orally. However, the experiments run subsequently show that some of the drug was dissolved into the water, either during the time when the food lay at the bottom of the tank or after it was egested or excreted by the fish in a still potent state. That these drugs affected the fish within a short time, through whatever means they became dissolved, is also evident. The evidence brought out by the later experiment showed, however, that the hormones are not stable under aquarium conditions for more than about three weeks, since after that time immature fish introduced into the tanks with the same water showed no effects whatsoever. Whether the hormone was destroyed by the microscopic population of the tank, adsorbed to the glass, or otherwise inactivated in some way is not known, but after that time it was no longer present in a form which had any perceivable effect on the fish. Further work is being done to determine the exact time when this inactivation takes place, and also, if possible, what the cause for the inactivation may be.

As to the effects of estradiol benzoate on the male, Cohen showed no figures on the development of either the control or the treated testes for eight-week-old fish, and therefore it is difficult to compare results at that age. In the present work, however, the testes so treated were slightly retarded in differentiation though not in size because of the administration of the hormone. Whether these effects are similar to those found by Cohen for an eight-week period is difficult to judge, because his descriptions did not cover that period.

Some support for the theory that different esters of the same hormone may bring about different effects was given by Grobstein (1942b), when he found that different esters of testosterone may show different effects on the regenerating anal fin of the platyfish. Even this paper, though, showed that all the esters used produced masculinization of the fin, as might have been expected. That a hormone and its ester should bring about diametrically opposed effects is unique. Grobstein also showed that the effects of these hormones is not to produce a normal gonopodium, but one that is imperfect. That evidence is substantiated here. In all cases there was produced a fin which was not precisely like the typical male gonopodium as it is seen in a normal adult animal. Even in those cases where the differentiated parts appeared to be almost normal, two differences in size were noted. The fin as a whole was smaller than the normal, and within this smaller fin the proportions existing between the length and width of the fin were changed. The 3, 4, 5 ray complex in each smaller fin was approximately one-third shorter than would normally have been found in a fin of the same width. The cause of this difference is apparently the result of differentiation of the fin beginning before it had time to grow to its full length, because of the relatively rapid action of the hormone. In the normal fish, the testis develops more slowly and therefore apparently controls the fin in such a way as to produce a lower amount of hormone until the fin has reached its maximum length, at which time the testis releases more hormone and differentiation takes place. This theory of hormone levels controlling the growth and differentiation patterns was postulated by Turner (1941b) and was adopted by Grobstein.

It should be established that under normal developmental conditions the young fish involved in these experiments would not have matured for about two months after the termination of the treatment, since they mature at approximately four to six months of age. Littermates of the experimental animals matured under conditions equivalent to those used in the experiments within these time limits, and averaged five months from birth to sexual maturity.

It can be assumed that a testis may be considered functional when it is producing spermatophores. Although no correlation has been found as yet to support this assumption in hormone-treated animals, it is always found that a normal functional male possesses spermatophores, while a non-functional male, otherwise normal, or an immature male, does not. Because of this evidence, it is assumed that the testes of the animals treated with pregneninolone, and the larger animals treated with estradiol were functional. Even if free sperm are produced under experimental conditions, the fish will not be sexually functional because of the necessity for transferring the sperm in a clump from the gonopodium of the male to the vent of the female. If this transfer is not carried out by way of the spermatophore, the sperm will presumably be lost in the water and fertilization will not result. Therefore, the important feature of the pregneninolone-treated testes was the large number of spermatophores present in both the acini and the duct. Since the normal testis at this age shows none of these features, the indication is that a great stimulation had occurred. Another feature to be mentioned is the difference in reaction of fish of the same age and size to the two hormones which produced stimulation of the testes. In the case of pregneninolone, the stimulation was a steady one, producing in every fish some sign of stimulation, the amount of growth and differentiation depending on the size and age of the fish. It was, however, never completely without effect. This may be seen from the sizes of the testes shown in Text-fig. 2 and Table II. Alpha estradiol, on the other hand, produces quite a different effect. In all the small fish, those below and including 18 mm., the effect was negligible. The testes appeared like normal control testes of the same age. When, however, the fish reached the size of 19 mm., the effect was different. The testes of the fishes of this size were immediately and greatly stimulated (see Pl. I, Figs. 4 and 5), and the testes resulting appeared to be functional, considering the great number of spermatophores present in the acini and duct.

As to the difference in size of the spermatogenic cysts present in the two types of treated animals, it is possible that the sudden arrival at a threshold level of hormone in the case of estradiol was responsible for a rapid differentiation of the gland, causing the smaller size of the spermatogenic elements. The pregneninolone-treated animals, which received a longer and steadier stimulation, were capable of producing cysts which were larger than those normally seen (see Text-fig. 3).

In order to suggest an explanation for the above effects and the others found in the present work, several assumptions must be made. First, it is well known that the liver of mammals inactivates steroid hormones which pass through the portal circulation (Biskind and Mark, 1939; Burrill and Greene, 1942; Cantarow *et al.*, 1943; Heller, 1940; Israel *et al.*, 1937; Segaloff, 1943; Talbot, 1939; Teague, 1941; Westerfeld, 1940). It is assumed that the same action takes place in the liver of teleosts. Some hormones, however, are inactivated more than others. Estradiol is inactivated more than estradiol benzoate because the benzoate ester protects the molecule from destruction. According to Heller (1940), the oxidation of the estradiols takes place at carbon 3 in ring A. Since the benzoate radical is attached at this position, its presence protects the molecule from oxidation (Segaloff, 1943). Therefore it can be assumed that the effective dose of estradiol, that is, the dose which produces the effects in the animal, is less than the effective dose of estradiol benzoate, if identical oral doses are given.

The toxicity of the hormones must also be taken into consideration. Plate V, Fig. 23, shows the appearance of the typical liver of an animal treated with pregneninolone. The cells are greatly enlarged and vacuolated and are presumably in a condition caused by the relatively great toxicity of the pregneninolone, which may be interpreted as a type of fatty change. Because of this toxicity, the liver, which at first probably rapidly inactivated the hormone, was rendered unable to do so, and the main portion of the hormone passed through the liver intact, producing a large effective dose and intense effects. The estradiol, which is partially inactivated, causes also a partial vacuolization of the liver, suggesting a cumulative effect on the liver, which results in an increase in effective dose. This eventually has an effect on the gonads.

If these hypotheses are true, they present new evidence for the action of abnormal quantities of metabolic substances on the liver, since till now the only concusive evidence for the inhibition of inactivation by the liver has been derived from work on experimental Vitamin B deficiency (Biskind and Biskind, 1941, 1942, 1943; Biskind, 1946).

A further assumption concerns the stage of growth and differentiation in which the gonads are found during the period of the experiment. During this period the testes are, for the purposes of this explanation, in a relatively undifferentiated state and not yet under the influence of the pituitary. Gonadotrophic hormones are known to be present in fishes (Scheer, 1948). There is evidence to support the above assumptions. The testes, as shown by Text-fig. 2, grow very little during the period of the experiment. They contain essentially the same elements at the end of eight weeks as they possessed about one week after birth. The ovaries, on the other hand, grow considerably during the same period, and yolk deposition is begun and progresses considerably. The ovaries and eggs are much larger at the end of the period than they were at one week of age. The growth of the gonads is known to be under the control of the pituitary (Matthews, 1939a).

If these assumptions are admitted, at least as possibilities, a hypothesis may be advanced as to the method by which the hormones produce their results in these experiments.

In the case of the testis, the first effective doses of pregneninolone were small because the substances were largely inactivated by the liver tissue. These relatively small doses stimulated the pituitary rather than inhibited it because of the smallness of the dose. The estradiol had a delayed effect because it continued to be inactivated for a longer period of time, and therefore needed a longer period of time in which to reach an effective dose. The dose which was effective in the case of estradiol was a cumulative one and required a longer period in which to operate and a larger animal on which to operate because of some type of threshold reaction. The estradiol benzoate went through the liver tissue undestroyed and reached the pituitary in doses large enough to cause an inhibition rather than a stimulatory effect. Thus the testis, which was not yet under pituitary control, showed little effect from the administration of this drug.

In the case of the ovaries, which were already under pituitary control, the effects were different. The smaller doses of estradiol and pregneninolone acted as partial inhibitors, shown by the partial inhibition of the eggs in these specimens, while the estradiol benzoate, again passing through the liver undestroyed, caused an almost complete inhibition of growth of the eggs.

To suggest an explanation for the action on the gonopodium is a more difficult problem. In both males and females, the effect on the gonopodium was similar. Pregneninolone stimulated at least some growth in all gonopodia, and all older animals treated for a longer period of time showed almost perfect transformation of the fin. Estradiol stimulated all gonopodia to a slight growth, and the largest ones to the same type of differentiation shown by the pregneninolone animals, though the differentiation was slightly less advanced. Estradiol benzoate had no effect on any of the animals. There are a number of hypotheses which may be advanced.

First, the gonopodium might be under purely genetic control. It is known that this is not true because the treated females showed differentiation to a gonopodium as readily as did the males.

Second, the ovarian hormone might inhibit the gonopodium. If we can assume that an inhibited ovary is producing little or no hormone, the above hypothesis cannot be true because under these conditions a greatly inhibited ovary would allow a better differentiated gonopodium than a partially inhibited one. The estradiol benzoate-treated ovary was inhibited to the greatest degree, but there was no gonopodium, while the animals which possessed partially inhibited ovaries formed well differentiated gonopodia.

Third, the reactions cannot be due to a non-specific reaction to steroids because the different substances produced different effects.

Fourth, if the reactions are due to the action of the fish testis hormone, or to an androgenic effect directly, it must be hypothesized that estradiol has a direct androgenic effect, while an effect based on dosage difference would be more plausible, since in mammals the substance has an estrogenic effect.

Fifth, control from the pituitary gland entirely could explain the effects in the males, where pregneninolone and estradiol stimulate the pituitary. In the females, however, the pituitary, according to the above assumptions, and based on its action on the gonads, inhibits the ovaries, and presumably would not at the same time stimulate the differentiation of a gonopodium. This of course assumes that the gonadotrophins secreted by both male and female pituitaries are qualitatively identical and stimulate the gonads of the animals in which they exist. This has been shown to be true for amphibians (Rugh, 1935).

Sixth, the theory that pituitary control plus male gonads or androgenic hormone cause the effects is the most nearly complete explanation. In this case, pregneninolone and estradiol stimulate the pituitary and therefore stimulate the gonopodium through the gonad. Estradiol benzoate inhibits the pituitary. Since the testis is not as yet under pituitary control, the testis shows no effects. No androgenic hormones are produced and the lack of these produces, in turn, lack of a gonopodium. In the females, however, an androgenic effect of the substances administered is necessary to explain the results. Pregneninolone and estradiol inhibit the pituitary and through it inhibit the ovary. The pituitary inhibition plus the androgenic effects of the hormones cause the differentiation of the gonopodium. Estradiol benzoate inhibits the pituitary, but, having no androgenic effect, does not cause the formation of the gonopodium.

A detailed cellular examination of the pituitary gland in these fish may reveal significant differences between controls and experimental animals, presumably involving the cells which secrete gonadotrophic hormones. This may furnish a partial explanation for the results described above and indicate whether the action may take place through the pituitary or is a direct effect of the hormones upon the gonads, as has been shown to happen in other animals (Nelson, 1937). A careful examination of the interstitial tissue of the testes may also aid in determining the possible effects of the hormones upon this tissue.

These hypotheses were constructed in an attempt to correlate the actions of the various hormones on the gonads and on the anal fin. Perhaps the effects on the two are entirely separate, however. The effect of the pituitary may be brought in to account for the effects on the gonads, but an androgenic effect of estradiol and pregneninolone would account alone for the effects on the gonopodium. It cannot be assumed, however, in view of the evidence brought out by estradiol benzoate treatment, that the effect is the paradoxical estrogen effect mentioned above caused by high dosage with estrogens. The effective dose of estradiol benzoate is higher than that of the others because it is protected in the liver. Therefore, under these conditions, one would be led to expect that it would produce a more definite effect than either of the other hormones. Since this is not true, some other hypothesis must be advanced to explain this effect. The other possibility which is most plausible is one in which the hormone has a directly androgenic effect.

The fact that the hormones which were used produced uniform results in spite of more than a tenfold range in dosage is an unusual finding. The toxicity which was found to be present with large dosages has also been found in mammals, but no sub-maximal results were found here.

It might have been useful also to treat the fishes with other benzoates as a control for the possible action of the benzoate ester exclusive of estradiol. The use of an inactive free compound with an active benzoate ester would be helpful in this work.

Turner (1941 a and b) brought to light various factors affecting the growth of the gonopodium. He stated that, first, the growth of the gonopodium depended on a certain low concentration of hormone and the differentiation of the fin depended on a higher concentration; second, that there existed certain dominances in the ray complexes which governed the differential growth of the various rays in such a way as to produce what we know as a complete gonopodium if the fin is left undisturbed; and third, that castration at any time during the growth of the gonopodium would stop its growth, while the administration of androgens thereafter would renew its growth. These findings on *Gambusia* have important bearings on the present studies. It was suggested above that the effect of estradiol was a cumulative one. This might account, on the basis of Turner's first statement, for the anal fin growth shown by the smaller estradiol-treated animals, where no differentiation was present. Pregneninolone caused an immediate and sustained effect on the gonopodium, suggesting that this hormone reached the threshold level almost immediately. Such a hypothesis would aid in explaining the effects on both testes and gonopodia.

As to the effect on the females, the hormones, as suggested above, may have had a direct effect on the fins.

The differential growth of the 3, 4, 5 ray complex was apparently governed by a low concentration of hormone. Dominance then shifted, according to Turner, so that the rays outside the 3, 4, 5 complex were subordinated to these three. This might explain the rapid growth of these rays in the young estradiol males and others in which a low level of hormone existed.

Castration with the effect of termination of growth of the fin, followed by androgenic restimulation, shows that the testis itself is not necessary for the growth of the fin, but that a hormone similar to that produced by the testis is required. This might aid in explaining the cases in which the females grew well-formed gonopodia.

The above hypotheses are far from clear and more work must be done in order to determine the explanation for these seemingly opposite and confusing effects. Hypophysectomy, castration and a combination of the two performed on animals which were later treated with hormones would aid in determining the mechanisms which govern these effects. Preparations for such work are going on now.

More work is necessary on the general problem of the differential effects of these two estradiol compounds. The exact stage when the differential effect on the male begins should be studied in more detail. Smaller dosages should be used in an attempt to discover a dose small enough to secure a less than maximum effect, as such an effect does not appear in the present work. Finally, an investigation into the differential effects of more compounds related to these should be carried out, since the exact effect of any one of them is now doubtful, whereas heretofore they have been used interchangeably, at least on experimental animals.

SUMMARY.

1. The experimental animal used was a strain of the platyfish in which males could be distinguished from females at birth as a result of a Y chromosome sex linked, spotted factor, whereas usually the sexes are indistinguishable until maturity, when the anal fin of the male is transformed into an intromittent gonopodium.

2. The hormones used were alpha estradiol, alpha estradiol benzoate and pregneninolone, a synthetic progestogen. These were administered by mixing the powder or oil solution with the food.

3. Pregneninolone exhibited a strong stimulating effect on the males, with precocious maturation of the testes and well-formed gonopodia. In females, development of the ovaries was inhibited, and gonopodia produced.

4. Estradiol benzoate was inhibitory on the testis and greatly so on the ovary. No gonopodia were produced.

5. Alpha estradiol had no effect on the testes of males under 18 mm. in standard length and produced slight growth of the anal fin. In males over 19 mm. in length, the testes were greatly stimulated and large, well-formed gonopodia were found. All females so treated showed ovarian degeneration and partial to nearly complete gonopodia.

6. Studies on the liver showed that pregneninolone and estradiol produced great vacuolization of the parenchyma cells and resulted in an organ which showed fatty changes, while the benzoate-treated livers appeared like those of the controls.

7. It is to be emphasized that although in the amniotes, estradiol and its ester are used interchangeably, in this species the two compounds produce diametrically opposed effects under the conditions of these experiments.

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EXPLANATION OF THE PLATES.

PLATE I.

Fig. 1. Testis of control male eight weeks of age. ×100.

Fig. 2. Testis of estradiol benzoate-treated male eight weeks of age. ×100.

Fig. 3. Testis of pregneninolone-treated male eight weeks of age. ×100.

Fig. 4. Testis of estradiol-treated male eight weeks of age. ×100. Compare with Fig. 3.

Fig. 5. Testis of estradiol-treated male eight weeks of age. ×100. Compare with Figs. 3 and 4.

PLATE II.

Fig. 6. Ovary of control female eight weeks of age. ×100.

Fig. 7. Ovary of estradiol benzoate-treated female eight weeks of age. ×100.

Fig. 8. Ovary of pregneninolone-treated female eight weeks of age. ×100.

Fig. 9. Ovary of pregneninolone-treated female eight weeks of age. ×100. Note scattering.

Fig. 10. Ovary of pregneninolone-treated female eight weeks of age. ×100. Note bilobed appearance of organ.

Fig. 11. Ovary of estradiol-treated female eight weeks of age. ×100. Compare with Fig. 9.

PLATE III.

Fig. 12. Ovary of estradiol-treated female eight weeks of age. ×100. Note large degenerating eggs and small abnormal eggs.

Fig. 13. Anal fin of control male. ×34.

Fig. 14. Anal fin of estradiol benzoate-treated male. ×34. Compare with Fig. 13.

Fig. 15. Anal fin of pregneninolone-treated male. ×34. Note almost complete differentiation of gonopodium. Compare with Fig. 13.

Fig. 16. Anal fin of estradiol-treated male. ×34. Compare with Figs. 13 and 15.

PLATE IV.

Fig. 17. Anal fin of control female. ×34.

Fig. 18. Anal fin of estradiol benzoate-treated female. ×34. Compare with Fig.17.

Fig. 19. Anal fin of pregneninolone-treated female. ×34. Compare with Figs. 15 and 17.

Fig. 20. Anal fin of estradiol-treated female. ×34. Compare with Figs. 16, 17 and 19.

PLATE V.

Fig. 21. Liver of control animal. ×960.

Fig. 22. Liver of estradiol benzoate-treated animal. ×960. Note similarity to Fig. 21.

Fig. 23. Liver of pregneninolone-treated animal. ×960. Note extensive vacuolization. Compare with Fig. 21.

Fig. 24. Liver of estradiol-treated animal. ×960. Note vacuolization—approaching but not equalling that of Fig. 23.